THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 262, No. 13, Issue of May 5, pp. 6089-6092, 1987
Printed in U.S.A.

Quantitation of the Interaction between Citrate Synthase and Malate Dehydrogenase*

(Received for publication, August 1, 1986)

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Formation of a bienzyme complex of pig heart mitochondrial malate dehydrogenase and citrate synthase in a buffered system is demonstrated by means of a covalently attached fluorescent probe to citrate synthase. Assuming 1:1 stoichiometry of the enzymes in the complex, an apparent dissociation constant of 10^{-8} M was calculated from fluorescence anisotropy measurements.

The effect of various metabolites on the interaction was tested. NAD^+, oxalacetate, citrate, ATP, and L(−)- or D(+)-malate had no effect on the association of the two enzymes, whereas α-ketoglutarate increased and NADH decreased it.

The interaction of mitochondrial citrate synthase with cytosolic malate dehydrogenase was found to be much weaker, whereas interaction of citrate synthase with another cytosolic enzyme, aldolase, could not be detected.

In kinetic experiments, the activation of malate dehydrogenase by citrate synthase was observed. The effect of pyridine nucleotides and α-ketoglutarate is discussed in relation to the direction of the metabolic flow of oxalacetate.

Increasing experimental evidence supports the idea, first proposed on theoretical grounds (1, 2), that proteins of the mitochondrial matrix are spatially organized. This arrangement may involve protein-protein interactions comprising sequential Krebs cycle enzymes (3, 4), malate-aspartate shuttle enzymes and Krebs cycle enzymes (5), and also interactions of matrix enzymes with proteins in the inner mitochondrial membrane (6, 7). Particularly interesting is the metabolic situation of citrate synthase. It is located at the point of influx of acetyl-CoA from glycolysis and fatty acid oxidation and seems to serve as a major site for the control of the Krebs tricarboxylic acid cycle (8). It is puzzling that the apparent concentration of its substrate, oxalacetate, does not appear to be sufficient for maintaining a flux through the cycle compatible with the observed respiration rate of mitochondria (1).

The interaction of citrate synthase and malate dehydrogenase may provide an explanation for this anomaly. The existence of this interaction has been demonstrated *in vitro* by coprecipitating the two enzymes in polyethylene glycol (3) and by investigating covalently immobilized enzymes (5, 9). In one of these studies (5), it could be shown that the fumarase-malate dehydrogenase complex binds alternatively citrate synthase and aspartate aminotransferase. Recently, the channeling of oxalacetate between malate dehydrogenase and citrate synthase in a polyethylene glycol-induced solid-state complex was observed (10). These results apparently support the hypothesis that the direction of metabolic flux of oxalacetate, either through the cycle or through the malate-aspartate shuttle, may be partially controlled by alternative complex formation of the aforementioned enzymes with channeling of oxalacetate. However, no indication has so far been presented as to how a dynamically interacting enzyme system such as this is controlled, *i.e.* which metabolites (if any) alter the system.

In the present study, we investigated the interaction of citrate synthase and malate dehydrogenase in an aqueous buffer. The fluorescence anisotropy of fluorescein isothiocyanate-labeled citrate synthase was measured as described previously (11–13). The interaction was characterized by determination of an apparent dissociation constant and by study of the effect of several metabolites thereon. We also investigated how complex formation influences the catalytic properties of the enzymes.

EXPERIMENTAL PROCEDURES

Materials—Ammonium sulfate suspension of porcine heart citrate synthase (EC 4.1.3.7) and 50% glycerol solution of mitochondrial malate dehydrogenase (EC 1.1.1.37) were from Boehringer Mannheim, whereas cytosolic malate dehydrogenase (EC 1.1.1.37) was from Sigma. Rabbit muscle fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), used after four-times recrystallization, was prepared according to Taylor *et al.* (14). L(−)- and D(+)-malate, acetyl-CoA, and α-ketoglutarate were from Sigma; NAD^+ and NADH were from Boehringer Mannheim; and fluorescein isothiocyanate-Celite was from Behring Diagnostics. All other chemicals were reagent-grade commercial preparations from Reanal (Budapest, Hungary).

Enzyme Preparations and Assays—Prior to experiments, the enzymes were centrifuged, dissolved in 0.05 M Tris/HCl buffer, pH 8.0, and filtered on a Sephadex G-50 column to remove traces of ammonium sulfate. Protein concentrations were determined by absorbance at 280 nm using absorption coefficients ($A^{1\%}$) and relative molecular weights of 2.9 and 67,000 for mitochondrial malate dehydrogenase, 13.1 and 67,000 for cytosolic malate dehydrogenase (15), 16 (5) and 96,000 (16) for citrate synthase, and 7.9 (17) and 160,000 (18) for aldolase, respectively.

Activities of citrate synthase (19) and malate dehydrogenase (20) were assayed as described previously. Spectrophotometric measurements were carried out on a Varian Cary 118 instrument at 25 °C.

Labeling of Citrate Synthase with Fluorescein Isothiocyanate—A crystalline suspension of 5 mg of enzyme was centrifuged, and the pellet was dissolved in 1 ml of 0.05 M Tris/HCl, pH 8.0. To this, 0.5 mg of fluorescein isothiocyanate-Celite was added; and after standing for 20 min at 4 °C, the sample was filtered on a Sephadex G-50 column to remove the unbound dye. It has been shown that the free

* This work was supported by the Veterans Administration, the United States Public Health Service, and the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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and bound dyes have the same absorbance (21). The extent of labeling was determined by measuring the absorbance of the treated enzyme at 490 nm using an absorption coefficient of 66,000 M^{-1} cm^{-1} (22). The average label of 0.35 ± 0.05 molecule of dye/enzyme molecule does not alter the activity of the enzyme. All fluorometric measurements were made on an Applied Photophysics SP3 instrument thermostated to 25 °C at excitation and emission wavelengths of 470 and 520 nm, respectively. Fluorescence anisotropy was determined as described previously (12).

Determination of the Dissociation Constant of the Enzyme-Enzyme Complex—For the quantitation of the interaction of fluorescein isothiocyanate-labeled citrate synthase with any of the nonlabeled enzymes (mitochondrial malate dehydrogenase, cytosolic malate dehydrogenase, and aldolase), the following procedure was used. The concentration of labeled citrate synthase was kept constant throughout while various amounts of the titrating enzyme (up to high molar excess) were added. After reaching a constant level (requiring less than 1 min), the anisotropy values of all points were measured. The dissociation constant was calculated by fitting the titration curve with a model assuming 1:1 stoichiometry in binding of the two enzymes (23). The following equilibrium was assumed to exist: M + C ⇌ MC (where M is malate dehydrogenase and C is citrate synthase). The titration curves were fitted by the function: $\text{anisotropy}_{obs} = ([C]a_C + [MC]a_{MC})/([C] + [MC])$ (where $a_C + a_{MC}$ are the anisotropy values of the free and complexed citrate synthases, respectively). a_C was measured in the absence of malate dehydrogenase, whereas a_{MC} was determined from the titration curves as anisotropy measured at the highest malate dehydrogenase concentration. At this point [C] = 0 and $\text{anisotropy}_{obs} = a_{MC}$. Citrate synthase and malate dehydrogenase concentrations were calculated from the total concentrations and assumed dissociation constants. The latter was varied to obtain the best fit for the experimental data. Calculations were performed with a Hewlett-Packard 9825A desktop computer equipped with a 9872A plotter.

In order to exclude the possibility that the fluorescent dye enhances the association constant, the labeled citrate synthase was displaced in the heterologous enzyme complex with unlabeled citrate synthase (Fig. 1, *lower*). Fig. 1 (*lower*) shows the anisotropy changes due to the labeled citrate synthase-malate dehydrogenase dissociation and the theoretical curve assuming a dissociation constant of 1 μM. The good correlation of the measured and calculated values suggests that, in the association of two enzymes, the fluorescent dye is not involved.

RESULTS

Interaction of fluorescein isothiocyanate-labeled pig heart citrate synthase and mitochondrial malate dehydrogenase was studied in 0.05 M Tris/HCl buffer, pH 8.0. As described under "Experimental Procedures," 3.5×10^{-7} M labeled citrate synthase was titrated with malate dehydrogenase in the concentration range of $0\text{–}3 \times 10^{-5}$ M. Increased anisotropy was found (Fig. 1), but fluorescence intensity was unchanged (not shown). These observations were independent of the extent of labeling. It may be concluded from the increasing anisotropy that the two enzymes interact in the concentration range used and from the constancy of fluorescence intensity that this interaction is not via the fluorescent moiety. Fitting the titration curve was done, taking the anisotropy of labeled citrate synthase to be 0.15 while taking that of the citrate synthase-mitochondrial malate dehydrogenase complex to be 0.225. The dissociation constant yielding the best fit was $1.0 \pm 0.4 \times 10^{-6}$ M in five experiments.

To address the question of specificity, the interaction of citrate synthase with two cytosolic enzymes was investigated in a similar manner (Fig. 1). One can see that the metabolically unrelated enzyme, aldolase, does not alter the fluorescence properties of labeled citrate synthase, indicating the lack of interaction. Although the increasing anisotropy with cytosolic malate dehydrogenase indicates an interaction with citrate synthase, the determined dissociation constant, $K_d = 1.5 \times 10^{-5}$ M, is an order of magnitude higher than that for the mitochondrial enzyme pair.

It was of interest to study the effect of various metabolites

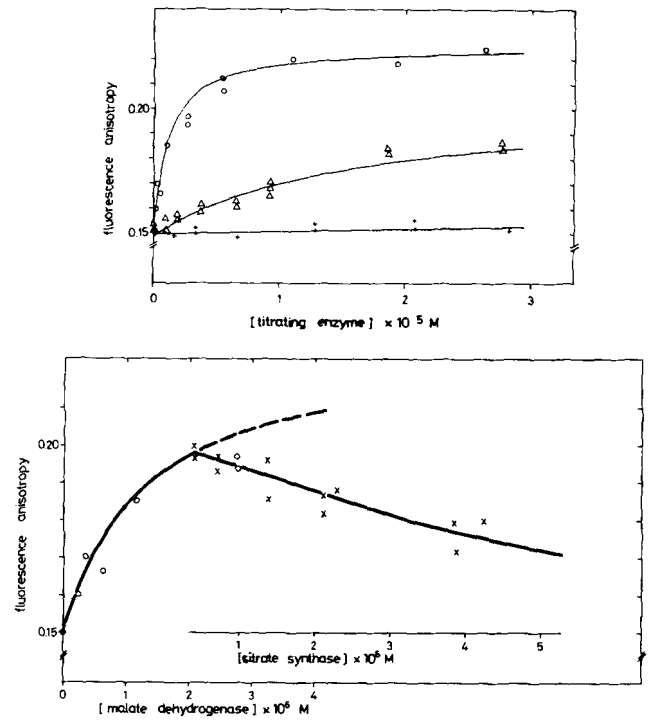

FIG. 1. *Upper*, change in fluorescence anisotropy of fluorescein isothiocyanate-labeled citrate synthase as a function of the concentration of titrating enzymes. Labeled citrate synthase (3.5×10^{-7} M) was titrated by mitochondrial (○) or cytosolic (△) malate dehydrogenase and by aldolase (+). Anisotropy values were determined after incubating the enzymes in 0.05 M Tris/HCl buffer, pH 8.0, at 25 °C until constant values were reached. Fluorescence intensity remained unchanged in time in all three cases in the whole concentration range. *Solid lines* are computer fittings assuming 1:1 stoichiometry of enzymes in the complex with apparent dissociation constants of 1.0×10^{-6} and 1.5×10^{-5} M for the complex with mitochondrial and cytosolic malate dehydrogenases, respectively. *Lower*, equilibrium displacement of the fluorescein isothiocyanate-labeled citrate synthase from the citrate synthase-mitochondrial malate dehydrogenase complex by nonlabeled citrate synthase. Labeled citrate synthase (3.5×10^{-7} M) was titrated by malate dehydrogenase and then displaced from the complex by adding nonlabeled citrate synthase. The *solid line* is the computer fitting assuming that the dissociation constant of the complex is unaffected by the label (dissociation constant is 1.0×10^{-6} M as in the *upper* portion). The sum of concentrations of labeled and nonlabeled citrate synthase is indicated.

on the interaction between citrate synthase and mitochondrial malate dehydrogenase. A set of titration experiments (Fig. 2) similar to that in the absence of metabolites was carried out, incubating the two enzymes in the presence of a 10^{-4} M concentration of the given metabolites. The calculated dissociation constants are summarized in Table I. It is seen that most of the metabolites exert no effect on the interaction, with the exception of α-ketoglutarate which enhanced and NADH which weakened it, as indicated by the altered dissociation constants. These changes are significant as seen from the experimental error of the measurements in Table I.

Having found that citrate synthase and malate dehydrogenase do interact in our buffer, we were interested in the kinetic consequences of the interaction. Previously, a change in catalytic properties of the interacting enzyme system was reported (9, 10). Under pseudo first-order circumstances, the mutual effect of citrate synthase and mitochondrial malate dehydrogenase was studied. Whereas the dehydrogenase exerted no effect on the synthase reaction, in the reverse case, we found an effect (Fig. 3). The transformation of oxalacetate appeared to be faster in the presence of citrate synthase, and

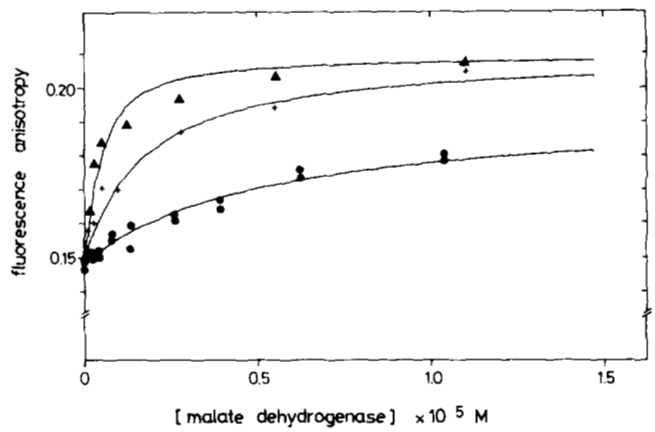

FIG. 2. **Effect of metabolites on the interaction of fluorescein isothiocyanate-labeled citrate synthase with mitochondrial malate dehydrogenase.** Labeled citrate synthase (1.5×10^{-7} M) was titrated by mitochondrial malate dehydrogenase in the presence of 10^{-4} M NADH (●) and at 4×10^{-7} M in the presence of 10^{-4} M NAD^+ (+) or α-ketoglutarate (▲). All conditions are as described for Fig. 1. Dissociation constants determined by computer fittings as described for Fig. 1 (also for citrate, ATP, and D(+)- and L(−)-malate (not shown)) are presented in Table I.

TABLE I

Effect of various metabolites on the apparent dissociation constant of the citrate synthase-mitochondrial malate dehydrogenase complex

Metabolite (10^{-4} M)	K_d
	10^{-6} M
None	1.0 ± 0.4
α-Ketoglutarate	0.2 ± 0.1
NAD^+	1.0 ± 0.2
NADH	5.0 ± 0.5
Oxalacetate	0.8 ± 0.3
Citrate	0.8 ± 0.1
ATP	1.0 ± 0.2
D(+)-Malate	0.9 ± 0.3
L(−)-Malate	1.0 ± 0.2

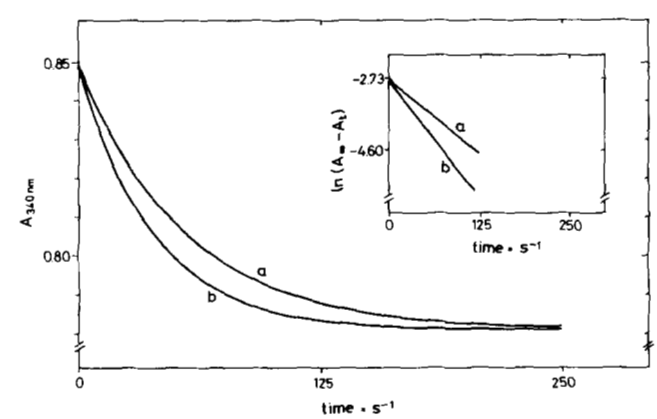

FIG. 3. **Kinetics of malate dehydrogenase-catalyzed reduction of oxalacetate in the absence (*curve a*) and in the presence (*curve b*) of citrate synthase.** Reactions were started by adding oxalacetate (1×10^{-5} M) to the preincubated mixture of 1.3×10^{-4} M NADH and 3.3×10^{-10} M malate dehydrogenase (*curve a*) plus 1.4×10^{-6} M citrate synthase in (*curve b*). The semilogarithmic plots (*inset*) indicate the pseudo first-order character of the kinetics since the starting value of oxalacetate concentration was below its K_m value (3×10^{-5} M in our case, not shown).

the effect seemingly increased with its concentration. Similar results were obtained at pH 7.0 (data not shown).

DISCUSSION

Our fluorescence anisotropy measurements show that the interaction of citrate synthase and malate dehydrogenase, predicted previously (1, 2) and revealed under some conditions (3, 9), exists in the bienzyme system reconstituted in an aqueous buffer. Applying the technique of fluorescence labeling has the advantage of sensitivity and applicability over a broad enzyme concentration range and also the possibility for determining quantitative characteristics of enzyme complexes. In the present work, we were satisfied with apparent dissociation constants (*cf.* Ref. 23) in the fitting of the experimental points, despite the fact that malate dehydrogenase is a dissociable enzyme (24).

Comparing the determined dissociation constant 1×10^{-6} M with the physiological concentrations of malate dehydrogenase and citrate synthase (4×10^{-5} M) (25), one might conclude that they are almost completely complexed in the mitochondrial matrix. Of course, the actual conditions existing *in vivo* may affect the strength of the interaction. The lack of interaction of citrate synthase with a metabolically unrelated enzyme, muscle aldolase, as a control is indicative of the adequacy of the fluorescence technique and of the specificity of interaction. Our finding, that citrate synthase interacts with cytosolic malate dehydrogenase much more weakly than with the mitochondrial isozyme, is consistent with the results of Halper and Srere (3). From a study of the interaction of citrate synthase with a large number of proteins (including muscle aldolase), these authors concluded the citrate synthase-mitochondrial malate dehydrogenase interaction to be physiologically specific.

The observed difference in the effects of pyridine nucleotides on the association/dissociation of this two-enzyme complex allows speculation on a possible physiological mechanism for regulation of a "dynamic channel" (*i.e.* metabolite channeling in transient enzyme complexes). Based on results presented here and elsewhere (26–29), it has become increasingly evident that dynamic (pair-wise) interactions represent a common form of enzyme organization *in vivo*. It is maintained that such transient (but metabolically specific) enzyme complexes endow a metabolic pathway with the usual advantages of the organized state (*e.g.* channeling (10)) while providing physiological variability and flexibility for control of metabolic flow (27). However, a crucial question concerning such dynamic channels is the manner of their regulation. So far, implications have been made that physiological fluctuations in regulatory ligands, substrates, etc. may govern the enzyme association (*e.g.* Ref. 30). With regard to the *in vivo* regulation of oxalacetate channeling, our results suggest a possible role of the NAD^+/NADH ratio. This is consistent with the existing view that the pyridine nucleotide redox state is a key energy-linked regulatory factor determining Krebs cycle flux (31).

Oxalacetate sits at an important metabolic branch point in mitochondrial metabolism. On the one hand, it may be transformed through the Krebs citric acid cycle during energy generation since, as discussed by Srere (1), channeling of oxalacetate between malate dehydrogenase and citrate synthase is vital for maintenance of sufficient respiratory flux. On the other hand, oxalacetate may be diverted toward other anabolic fates. It can enter the malate-aspartate shuttle (it appears that heart muscle relies heavily on this shuttle) (32), or it may be utilized in gluconeogenesis (in liver and kidney). The lack of an effect of malate dehydrogenase on citrate synthase activity might lead one to dismiss the physiological

importance of the interaction. However, the constraints (non-physiological conditions must be used) of the enzyme assay make any physiological interpretation of that experiment difficult at best. In other experiments on an intact system (33), the physiological advantage of this interaction is demonstrated.

Since NADH affects the strength of the association between malate dehydrogenase and citrate synthase, the degree of enzyme interaction (and hence the oxalacetate-citrate flux) may be readily controlled by changes in the NAD^+/NADH ratio. Accordingly, the NAD^+/NADH ratio may serve a dual role. In the first case, it is a major determinant in the energy-generation flux in the Krebs cycle by virtue of the regulatory effects on various enzymes therein (31). In addition, it may function in the control of metabolic flow of carbon between catabolic and anabolic fates.

The effect of α-ketoglutarate is more difficult to explain physiologically. This Krebs cycle intermediate also has alternate metabolic fates, *e.g.* glutamate formation and the malate-aspartate shuttle. Activation of the dynamic channel between malate dehydrogenase and citrate synthase by α-ketoglutarate is consistent with the necessity of maintaining balanced fluxes between α-ketoglutarate dehydrogenase and citrate synthase (31). However, additional studies are necessary to substantiate this regulatory modality.

In conclusion, this system may be a rather unique example reported so far where divergent metabolic fluxes are regulated by direct control of dynamic (pair-wise) enzyme interactions.

Acknowledgments—Penny Perkins assisted in manuscript preparation.

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